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14. ABSTRACT
The phase I SBIR grant had two primary objectives. The first objective was to demonstrate the fabrication of large arrays of individually addressable, nanometerscale ultramicroelectrodes (nanodes). The second objective was to develop model immunochemical assays that can be monitored by electrochemical means for a wide range of chemical and biological warfare agents. Attaining these technical objectives would demonstrate the feasibility of using integrated circuit sensor devices for multiplexed assays. We developed the CombiMatrix ArrayChip for use in biowarfare agent detection and determined the feasibility of monitoring multiplexed assays on the chip surface using new electrochemical techniques that are in development.

15. SUBJECT TERMS
Biosensors, microarrays, redox based electrochemcial detection, immunoassays, toxins.

CombiMatrix Corporation

Nanode Array Sensor Microchips

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Phase I Final Report

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List of Abbreviations Used

BG	Bacillus *Globigii*
SA	streptavidin
Au	gold
BSA	bovine serum albumin
MAb	monoclonal antibody
PAb	polyclonal antibody
α	anti
TR	Texas Red
F	fluorescein
b or B	biotin
TR-BG	Texas Red-labeled BG spores
TR-Ab	Texas Red-labeled antibody
b-Ab	biotin-labeled antibody
b-NHS-PEG	biotinylated PEG-N-hydroxysuccimide ester
b-LC-NHS	biotin-long chain-N-hydroxysuccimide ester
b-NHS	biotin-N-hydroxysuccimide ester
F-NHS	fluorescein- N-hydroxysuccimide ester
PEG	polyethylene glycol
μ	micron
Mw	molecular weight
pg	picograms
fmol	femtomol
pmol	picomol
μg	microgram
pC	picocoulombs
pL	picoliters

Scope of Project

Immunochemical assays are highly selective and are in current use for monitoring chemical and biological warfare agents. The size of the biological agent varies from several hundred molecular weight (saxitoxin) to the use of spores (anthrax) and cells. Traditionally, a whole host of methods have been used (flow cytometry to immunoassays) for the analysis of the agent present. Some of these assays are time consuming and in some cases labor intensive. Moreover, high throughput assays for analyte samples also becomes problematic. Thus newer, more sensitive, higher throughput and multiplexed assay techniques needed to be developed. To accomplish this the assay platform as well as the analyte detection format needs to be altered.

The phase I SBIR grant had two primary objectives. The first objective was to demonstrate the fabrication of large arrays of individually addressable, nanometer-scale ultramicroelectrodes (nanodes). The second objective was to develop model immunochemical assays that can be monitored by electrochemical means for a wide range of chemical and biological warfare agents. Attaining these technical objectives would demonstrate the feasibility of using integrated circuit sensor devices for multiplexed assays. We developed the CombiMatrix ArrayChip for use in biowarfare agent detection and determined the feasibility of monitoring multiplexed assays on the chip surface using new electrochemical techniques that are in development.

The use of electrochemical methods for detection with immunochemical assays will enable porting these techniques to arrays of microelectrodes. CombiMatrix is in the early stages of methods development that will enable immobilization of different assays at different electrodes in an array of individually addressable electrodes (1). We have shown that these electrode arrays may be used to carry numerous different immunochemical assays.

The electrochemical technology concerning the arrays of nanometer-scale ultramicro electrodes (nanodes) have great potential for improving the detection limits, selectivity, versatility, and portability of sensor devices that detect agents used in chemical and biological warfare. Arrays of nanodes greatly improve signal-to-noise and detection limits (2). By operating within a semi-hemispherical diffusion regime, nanodes enhance the ratio of faradic current to capacitive current by orders of magnitude over conventional electrodes (2). This enables ultra-high scan rate voltammetry and a very rapid establishment of a steady state in chronoamperometric experiments. Further, arrays with individually addressable nanodes enable spatial multiplexing of highly selective immunochemical assays for a plethora of different chemical and biological warfare agents on a single integrated sensor device.

To further improve the sensitivity and extend the range of electrode diameters that can be fabricated on existing CombiMatrix electrode array hardware into the nanometer regime,

we initiated a project to develop the ultramicroelectrodes. Conventional excimer laser lithography can be used to manufacture devices with feature sizes as small as 180 nm. CombiMatrix will combine state-of-the-art lithographic techniques with low cost CMOS device fabrication to produce analog VLSI devices with arrays of individually addressable nanodes.

Integrated CMOS sensor devices that can perform numerous simultaneous assays for the presence of a variety of chemical and biological warfare agents can fill an important role in field deployable threat assessment tools. The CMOS sensors enabled by the technologies described in this proposal are i) small, ii) have very low power consumption characteristics, iii) perform simultaneous assays for numerous chemical and biological warfare agents, iv) have low levels of detection, v) are highly selective, and vi) are low cost. This portion of original proposal will be further investigated in the phase II SBIR.

CombiMatrix ArrayChip™ Technology

A) Current Chip Technology

CombiMatrix designs and produces integrated circuits that are high-density arrays of individually addressable electrodes. Electrode arrays have been produced with platinum, gold and silver metallization. CombiMatrix can electrode arrays with any desired metal in any pattern. Electrode diameters can be made as small as 180 nanometers when patterned with excimer laser lithography.

CombiMatrix uses CMOS technology to create analog VLSI electrode array devices with parallel addressing for selecting and controlling individual electrodes in the array. Each individual electrode cell in the array includes an SRAM cell, a bank of transistor switches to select from several voltage or current sources, and quality control and validation hardware. CMOS devices have significant advantages for applications that require portability and low power consumption.

The first generation CombiMatrix electrode array chip, the CME9608I, was delivered in 1996. Figure 1 shows a photomicrograph of a portion of the CME9608I chip. Using the CME9608I, CombiMatrix has developed an extensive suite of hardware and software for controlling multiplexed electrochemistry and quality validation tasks on electrode array chips with individually addressable electrodes. The CME9608I has 1024 electrodes that have individual voltage control. This chip is based on an inexpensive 3μ CMOS process. CombiMatrix also has two additional chip designs, the CME9812CV and the CME9810HD. The CME9812CV is based on a 1μ CMOS process and

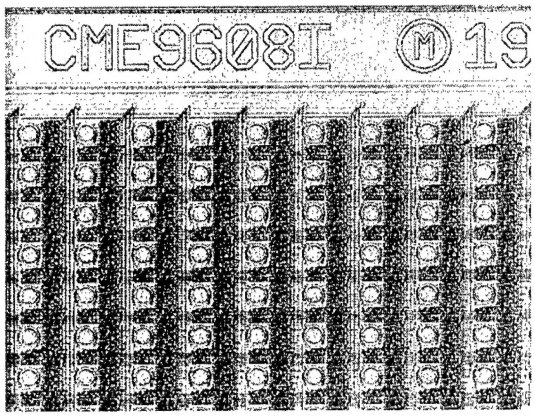

Figure 1

has an array density of over 10,000 individually addressable electrodes per cm^2, each of which has individual voltage control and current sourcing. The CME9810HD is based on a 0.36μ CMOS process and has approximately 500,000 individually addressable electrodes per cm^2, each of which has independent voltage control.

CombiMatrix also has developed proprietary on-chip integrated circuitry that is designed to prevent and to monitor contamination of CMOS circuitry by ions diffusing into the integrated circuit from electrolyte solutions. The inherent sensitivity of integrated circuitry to ion contamination arises because transistor junctions are doped with ions at nanomolar concentration levels. As a result, diffusion into an integrated circuit of as little as one part per billion of contaminating ions from an electrolyte solution in which the chip is immersed will destroy the integrated circuit. Figure 2 shows the shift in threshold voltage of CMOS transistor structure designed to monitor ion contamination levels within a CME9608I chip. Shifts of over 2 V occur within twenty minutes of exposure to an aqueous 0.1 M sodium phosphate solution at 46°C. Voltage shifts of this magnitude will destroy an active device. However, using the proprietary circuitry that CMX has developed, CME9608I devices have been operated successfully for over 500 hours while immersed in a 1M aqueous electrolyte solution.

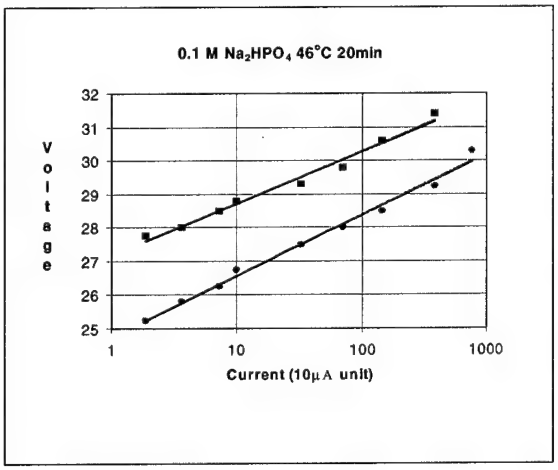

Figure 2

B) Membrane Technology

CombiMatrix has developed technologies and materials for coating its chips with permeable membranes. It has also developed methodologies and chemistries for immobilizing antibodies, enzymes and antigens within these membranes at selected locations. CombiMatrix demonstrated one of its immobilization methods at the National American Chemical Society meeting held in Boston, MA in August 1998 (1). Using electrochemically-generated reagents, biotin was immobilized over selected electrodes. The quantity of immobilized biotin can be controlled coulometrically by adjusting the total charge passed through selected electrodes.

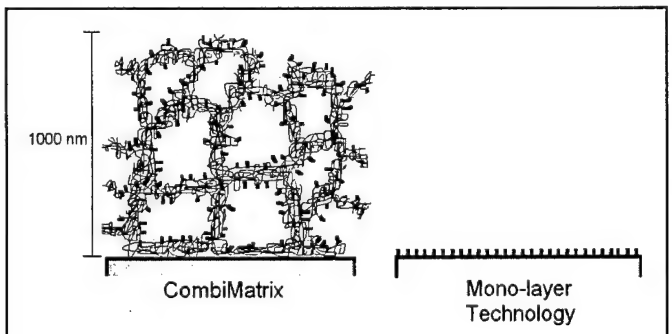

Figure 3

Any biomolecule that can be tethered to a biotin can be immobilized at selected locations on CombiMatrix chips using this technology. CombiMatrix membranes are formed from hydrated and biocompatable carbohydrate materials. These materials are

extremely porous. Consequently, large molecules,
such as antibodies, have facile access to the membrane interior (Figure 3). The full spectrum of immunochemical assay formats can be ported to the CombiMatrix chip platform.

C) Analytes Investigated

Chemical and biological warfare agents range from small molecules, such as soman, to spores and cells. Current levels of detection (LODs) for many of the smaller molecules are on the order of ng/test (3-6). LODs for larger molecules, such as ricin, are in the range of pg/test (3-6). Cells and spores can be detected at levels ranging from 10-1000/test (7-10). All of these tests are based on immunochemical methods.

Because of DoD interest in the various molecules, we focussed on the extremes of the molecular weight range of the various analytes: Saxitoxin (Mw = 301.2) (Fig. 4) to spores (3 μ in diameter). For the initial feasibility studies in phase I, these were the analytes most applicable and readily available. Saxitoxin antibody was obtained from Custom Monoclonals International, located in Sacramento, CA. BG spores were available from ERDEC BIDS team. Saxitoxin is commercially available and BG sores antibodies were also provided by the BIDS team.

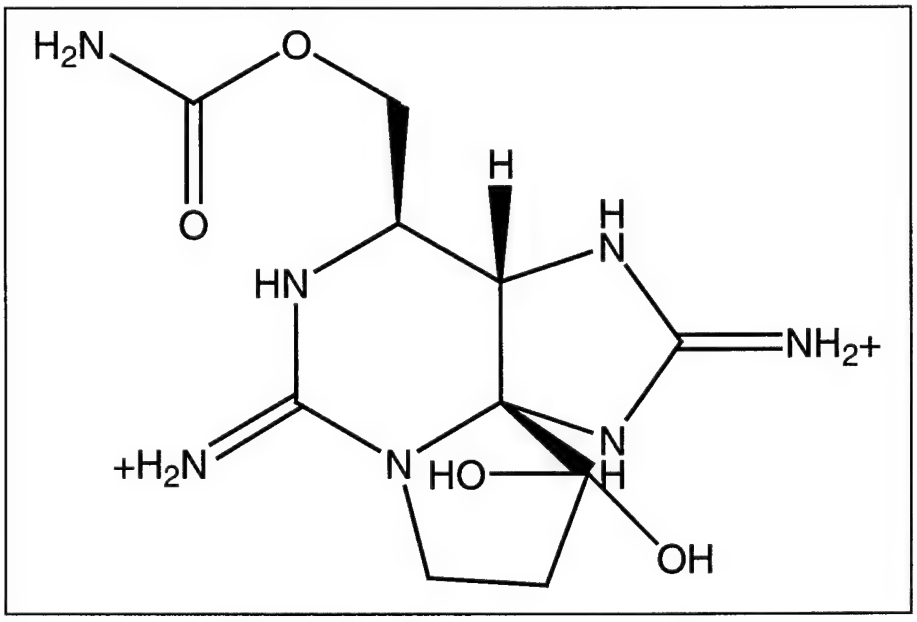

Figure 4
Structure of Saxitoxin

D) Assay Methodology

In general, immunochemistry formats in current use for detection of chemical and biological warfare agents are either competitive or sandwich assays. Competitive assays have advantages for small and medium sized antigens. Sandwich assays have advantages for larger molecules or spores and cells (7-11) and usually provide much lower limits of detection.

Saxitoxin is a useful compound for a model assay system for small molecules that have only one epitope. A competitive assay format is preferred in this case. Immobilized saxitoxin will form an antibody-antigen complex with the appropriate antibody. Exposure of the immobilized antibody-antigen complex to a solution containing free saxitoxin will cause competition between the free and the immobilized antigen for the antibody. This is directly observable as a loss of signal from the antibody (4,5). This assay format is quite often called an indirect detection method as the signal is generated only in the absence of analyte present. A diagram describing the assay format is given in Fig. 5.

B. *globigii* is a good model of large biological warfare agents such as anthrax spores. A sandwich format is preferred in this case. Immobilized antibodies to B. *globigii* will capture spores from an analyte solution. Free antibody that is enzyme or fluorescently-labeled is added to the immobilized spores on the chip. Presence of the spores is directly observable as a signal from the labeling sandwich antibodies (7,8). The assay format is such that signal will only be observed, if analyte is present. A diagram describing the assay format is given in Figs. 6,7.

E) Detection Methods

In phase I , we were to provide assays based upon redox active tags bound to antibodies. However, the assay procedures were to be based upon corroboration by detection via a more conventional method, such as fluorescence. In this case, the antibody needed to be labeled with fluorophores such as fluorescein or Texas red.

Fluorophores can readily be attached to proteins such as antibodies, or to proteins found on cell or spore membranes via NHS chemistries. Specific protocols are typically provided by the manufacturers, such as Molecular Probes or Molecular Devices. Typically, a DMF solution containing the fluorophore NHS ester is freshly prepared and used within an hour of preparation. A portion of the DMF solution is added slowly, in aliquots, to the protein in phosphate buffer between the pH range 7.5 to 8.5. The quantity of DMF added to the protein solution is usually 1% or less (v/v). Additionally, the labeling range requires that the NHS ester be present in a 5-20 molar excess. Typically, the protein concentration is 1 mg/mL, but that value can be lower. More detail will be given in the results section for each analyte.

Fluorphore detection is accomplished using an Olympus BX60 epifluorescent microscopy system that is modified for imaging chips. Images are acquired using integrating or intensified CCD cameras. Acquired images are captured digitally using commercial video capture cards and software that was developed internally by CombiMatrix. CombiMatrix has also developed a large set of image analysis software for quantifying fluorescent micrographs and gathering quality control statistics on its assays.

Presently CombiMatrix uses the chip to generated electrochemical reactions at specific electrodes. Conversely, the chip should be able to detect electrochemical (redox) reactions at specific sites on the chip surface. To this end, we require an enzyme based redox chemistry that can be linked to antibody or any protein. Typical enzymes that can be used for this purpose are β-galactosidase, glucose oxidase, and horseradish (or soybean) peroxidase. For the phase I SBIR we investigated the use of β-galactosidase, but in the interim period before phase II funds arrive, we are investigating the use of soybean peroxidase (about 50,000 Mw) in our bioassays.

Antibodies used for immunochemical assays can be modified with redox active moieties. These redox active tags can be detected directly at an electrode or indirectly via a mediated detection scheme. Mediated detection is preferred with antibodies because they often can denature or lose activity upon direct contact with an electrode. Enzymes are a very useful class of redox active modifiers. Enzymes will often turn over 10^4-10^6 molecules of substrate per second, which greatly amplifies their electrochemical signal.

β-Galactosidase has proven to be a useful and robust enzyme for modifying antibodies. The substrate for β-galactosidase is 5-bromo-4-chloro-3-indoyl-β-D-glactopyranoside (X-Gal) (Fig. 7). X-Gal is cleaved by the enzyme to form galactose (Gal) and 5-bromo-4-chloro indoxyl (X). X can undergo a one electron oxidation (to form an indigo compound) and allow a redox mediator scheme to monitor its production by β-galactosidase; an enzymatic outline is given in the next section. The most common redox mediator scheme for oxidizing X is ferri/ferrocyanide. This enzymatic reaction is preferred because β-galactosidase is extremely stable and the reaction proceeds at neutral pH.

Assay Procedures

A) ArrayChip Preparation

ArrayChips were prepared for use by coating the surface of the CHME 96081 chips with proprietary carbohydrate-based matrix. These matrices are approximately 1-2 microns thick. Proprietary electrochemical methods were used to immobilize biotin within the interstitial regions of the matrix over selected electrode locations. The programmed patter allows straightforward analysis of the assay signals.

A variety of biotin containing compounds may be covalently bound to the membrane. At CombiMatrix we have traditionally used a short chain biotin-containing compound. This has been quite adequate for most uses. However we have found that a longer chain biotin-containing compound would be necessary in order to bind the larger molecules (distance from the surface is important. Thus for most of our studies we have incorporated the use of a biotin compound that contains close to 70 PEG units. This allows the biotin to be a considerable distance from the membrane.

With the biotin covalently attached to the membrane, streptavidin can readily be bound via one f the four biotin-binding sites that are available.

B) Saxitoxin Assay

Saxitoxin is a small molecule (as per Fig. 4) with a single epitope. Hence a competitive assay protocol was formulated (Fig. 5) whereby a saxitoxin molecule would be bound to the membrane. The proposed and executed format was with saxitoxin covalently bound to the streptavidin.

Streptavidin (Vector Labs, Burlingame, CA) was succinylated with succinic anhydride under basic conditions. The modified streptavidin was separated from the reaction by-products and salts using a 100 uL spin column (Clontech, Palo Alto, CA) . The sample was freeze-dried for storage. A fraction of the succinylated streptavidin was mixed with saxitoxin in phosphate buffer at neutral pH. Coupling of saxitoxin to the succinylated streptavidin was accomplished using a water-soluble carbodiimide. The sample was purified once more using a 100 uL Clontech spin column.

Labeled anti-saxitoxin antibody was obtained as follows: Rabbit serum containing polyclonal anti-saxitoxin antibody was obtained from Custom Monoclonals International, Sacramento, CA. IgG was purified using a protein G column (Amersham Pharmacia); purified IgG was eluted at pH 4.5. The antibody was labeled with Texas Red-NHS using the procedures outline for "antibody labeling" in the Molecular Devices' Threshold manual. TR-NHS reaction by-products were removed from the TR-labeled antibody using a Clontech spin-column.

The assay procedure was as follows: The ArrayChip was coated as described above. After biotinylation, the chip was soaked in a solution containing the streptavidin linked saxitoxin. The chip was washed and then placed into a solution containing the TR-labeled rabbit anti-saxitoxin antibody. After this incubation, the chip was washed and the fluorescence measured.

A dilution series for saxitoxin on the ArrayChip was prepared as follows: The ArrayChip was coated with our biomembrane as noted in earlier reports. At this point reagents for the incorporation of biotin-polyethyleneglycol (b-PEG) onto the membrane surface were added and the b-PEG was electrochemically attached. The difference in the present study

was that the current to designated electrodes was set to varying time intervals. The current "on time" is directly proportional to the quantity of biotin moiety that is covalently attached to the membrane. Consequently, this affects the quantity of streptavidin-saxitoxin that can be bound to the membrane under saturation conditions. The binding of Texas Red-labeled anti-saxitoxin antibody (PAb) to the membrane-bound saxitoxin can provide a "dilution series".

C) BG Spore Assay

The antibody used in all our BG detection experiments was derived from goat serum provided by the BIDS team. The polyclonal goat anti-BG antibody was obtained from the goat serum after purification and isolation of the IgG from a protein-G affinity column (Amersham Pharmacia). Approximately 6 mgs of IgG were obtained from 2 mL of serum. One mg of purified IgG was labeled with F-NHS as per procedures described above. Another mg of the antibody was labeled with b-PEG-NHS (also tried b-DNP-NHS). A ½ mg antibody fraction was also labeled with TR-NHS and purified on a Clontech spin column.

BG spores were tagged with TR label based upon the covalent linkage of TR to proteins on the spore surface containing free amino groups. This was accomplished in a manner similar to the labeling of proteins. However, a large excess of TR-NHS was used and the hydrolyzed (not reacted with protein) TR was removed by at least 5 washings with phosphate buffer, pH 7.0. The spore washing was accomplished by centrifugation of the spores in a 1.5 mL Eppendorf microtube and the supernatant was removed. More phosphate buffer is added, the spores vortexted, and the sampled centrifuged. The process was repeated until the supernatant was clear.

For the detection of TR-labeled spores (Fig. 6), a site-specific b-PEG coated chip was incubated with a solution containing strepatvidin. The chip was then extensively washed and placed into a solution containing b-PEG-anti-BG antibody. After rinsing the chip, the Texas Red labeled-spores were layered onto the chip and placed into a humidifier for 1 hr. The chip membrane was then washed fluorescence measured taken on the epifluorescent microscope.

For the BG spores sandwich immunoassay (Fig. 7), the initial procedures were the same as above. However after the chip had been soaked with biotinylated antibody, unlabeled spores were layered onto the chip (1 hr. incubation). After the chip was washed, TR-labeled goat anti-BG antibody was added and allowed to soak for an hour. The chip was washed with phosphate buffer and water before fluorescence measurements were taken.

D) Assay Interfering Substances

The specificity and performance of any assay depends upon assay NSB, assay and specificity (no cross-reactions), and assay interference. To check for assay interference a

number of substances need to be added to the assay mixture to determine if those materials add or detract from the assay performance.

To this end, we decided to investigate numerous substances that could interfere with the saxitoxin and BG assays. In both cases compounds were chosen that were similar in nature to the analytes that were being tested. For saxitoxin, we chose to use brevetoxin (another marine toxin), ricin (an extremely toxic glycoprotein), and digoxin (a naturally occurring host of compounds including digitalis). For BG spores, we chose pombe (yeast) and salmonella.

Fluorescence-Base Immunoassays (Results)

A) Saxitoxin

Assay format for saxitoxin was followed as described above. The biomembrane was biotinlyated in a checkerboard pattern above selected electrodes. The on-electrodes received the same quantity of current for a set time period (uniform labeling), resulting a uniform pattern across the chip. The chip was then soaked in SA-saxitoxin followed by TR-labeled rabbit anti-saxitoxin antibody. Thus, TR-based fluorescence should be visible only in areas where SA-saxitoxin would be bound. As a control the lower portion of the biotinylated chip was soaked in SA-F. Thus we observed fluorescein-specific fluorescence in this area and TR-specific fluorescence in the other portions of the ArrayChip.

The fluorescence micrograph of a section of the ArrayChip that contained SA-STX is shown in Fig. 8. Since this picture was taken with the TR-filter, the observed fluorescence must result from TR-labeled Ab bound to that site. Note the uniform fluorescence emanating from the Chip; a clear indication that the immunochemistries have indeed worked.

To check for interfering substances, we modified the assay such that the antibody was pre-incubated with the interfering substance (at 1 mg/mL) before the solution was spotted onto a section of the chip containing bound SA-STX. The fluorescence intensity was measured using the CombiMatrix software and the data is presented below in Table I. The values are given in percentages with respect to the intensity observed without the interfering substance being present.

TABLE I

Assay	Relative Signal
Saxitoxin Control	100%
Digitoxin	84.6%
Brevetoxin	102%
Ricin	81.5%

Ultimately a standard curve needs to be developed for the saxitoxin assay with unknowns to be tested. As a starting point, a saxitoxin "dilution curve" can be provided. This can be accomplished via the fixed current, time dependent, biotinylation of the membrane (see above). After the biotinlyation has been accomplished, the chip is soaked in SA-STX, washed and placed into a solution containing TR-labeled rabbit α-STX antibodies. The ArrayChip is then washed and the fluorescence measured. The results are shown in Fig. 9. The curve is linear for the most part, except at very high times (over 15 minutes). This could result form fluorescence quenching due to fluorophores being in close proximity. This would not be a problem, if an alternative mode of detection were used.

B) BG Spores

The simple method for the determination of whether spores can be detected on the chip, would be to use F-BG spores. In this way, only a single biotinylated antibody needs to be used. The antibody is layer onto the chip and acts like an anchor for the spores. The spores are then layer on, allowed to incubate and the excess, nonbound spores removed, with gentle washing. For future use, microfluidics may be an alternative to layering. Results can be seen in Fig. 10 for the capture of TR-BG on a uniform biotinlyated chip containing SA and biotinylated goat anti-BG PAb. Note that the checkerboard pattern relating to the electrodes that were turned on.

For this assay, formaldehyde treated salmonella and pombe (yeast) were used (at 1 mg/mL) to ascertain whether another cell system could interfere with the performance of the BG assay. The results are given in Table II, as a percentage relative to the performance (fluorescence intensity) of non-spiked assay. In all cases it would appear that the performance of this assay is unhindered by the cells added to this mixture.

TABLE II

Assay	Relative Signal
BG Control	100%
Yeast	99.6%
Salmonella	89.6%

A BG detection curve (loading study) was prepared based upon the binding TR-BG spores to the biotinylated membrane. Diluted TR-BG spores (in 0.5 uL quantities) were placed over sections of the chip and allowed to bind. The chip was washed and the intensity measured. The approximate quantity of spores bound to the electrode-membrane surface was calculated based upon the solution concentration of the spores and the relative area of electrodes (%) versus the entire chip surface. The dilution results are shown in Fig. 11. This appears to be a linear plot with the limit of detection at about 50 spores.

A more realistic assay for BG spores would be to use unlabelled spores and one antibody to capture the antibody while a second, fluourescently-labeled antibody would be used to detect the spore. This would be called a sandwich assay as depicted in Fig. 7. The only drawback under current conditions is that we have only a single type of antibody (PAb) available---targeting identical BG-epitopes. A more practical solution would be to obtain a MAb for the BG spores while using the PAb for detection.

However for the current immunosandwich assay , we only had the one PAb available, so one portion of the Ab sample we biotinylated while the second portion was TR-labeled. In order to avoid competition for the same epitopes by these labeled antibodies, we used a sequential layering sequence. After the Biotin and streptavidin had been placed on to the chip, the chip was soaked in a solution of biotinylated goat-α-BG antibody for at least one hour. Following this, the chip was extensively washed and BG spares were layered onto the chip in a humidifier. After about one hour the chip was gently washed and TR-labeled goat anti BG was layered on the chip. After a one hour incubation, the chip was rinsed very gently and fluorescence reading taken. The results are shown in Fig. 12. The sandwich sassy does provide remarkable results. However, the assay performance could be improved if a biotinylated monoclonal antibody were used for the capture of the BG spores.

A BG standard detection curve was also developed using the assay procedures given above. The results are shown in Fig. 13. The procedure used for the calculation of the numbest of BG spores per spot is the same as that used for TR-BG. Note the near linear response over the range 0-1,500 spores. The LOD for this curve is approximately 100 spores. Again, the assay performance could be improved if a monoclonal antibody were used to capture the spores.

Electrochemical Methodology

A) Biochemical Redox Reaction

As mentioned in the outset, we indicated using one or more electrochemical reactions to promote the detection of our multiplexed ArrayChip with a method other than fluorescence. A "site specific enzyme amplified electrochemical detection" protocol has been used for ELISA experiments and should be the best for our system. In this case, the enzyme (conjugated to the antibody) would hydrolyze a substrate that could be detected electrochemically at the electrode surface. There are a host of enzymes that fit this capacity: These include horseradish peroxidase, glucose oxidase and b-galactosidase.

Each one of these enzymes has some positive and negative features. We chose β-galactosidase as our first attempt to find a system that would work with our chip. Mainly, the electrochemical reactions for this system had been carried out and the enzyme was stable at neutral pH.

An alternative to fluorescence based detection methods would be to use "site specific enzyme amplified electrochemical detection". The protocol would be the same as that used in traditional ELISA assays except that an enzyme conjugate is used which hydrolyses a substrate in a reduction/oxidation reaction. There are numerous enzymes that would function in this capacity, but we chose to begin our investigation of this assay format using β-galactosidase, an exo-glycosidase. The normal substrate for β-galactosidase would be "terminal β -galactopyranoside", but any chromophore (ortho-nitrophenol or 5-Br-4-Cl-indoyl) or fluorophore attached to β -galactopyranoside would also work. To this end we decided to investigate the use of 5-Br-4-Cl-indoyl- β -galactopyranoside (X-Gal) as our substrate for β-galactosidase because the enzymatic reaction produces a product that is electrochemically active, as shown in Fig. 14.

The enzymatic reaction produces the indoyl species that undergoes a dimerization (the enol tautomeric form) and then proceeds to undergo another 2-electron oxidation for the formation of a blue-indigo dye. These oxidative transformations are mediated by a reversible redox couple that acts to shuttle electrons from the electrode to indoxyl moieties proximate to the electrode. The ferrocyanide/ferricyanide redox couple was chosen as a mediator. This Fe2+/Fe3+ redox couple is well behaved in most circumstances and has sufficient free energy to drive rapid oxidation kinetics.

B) ArrayChip Cyclic Voltammetry Results

In order to determine if the ArrayChip can indeed monitor the electrochemical enzymatic reaction described above, we inaugurated several experiments involving the "cyclic voltammetry" studies of the Ferrocyanide/Ferricyanide couple with (and without) β -galactosidase and substrate present, using the ArrayChip and a standard electrode (Ag/AgCl reference).

Cyclic voltammograms obtained from a platinum electrode on the ArrayChip are shown in Fig. 15. A PAR 283 potentiostat was used to obtain the data. Both reference electrode and counter electrode were external to the ArrayChip. The reference electrode was Ag/AgCl and the counter electrode was a platinum wire. A 0.1 M aqueous phosphate buffer at pH 7.2 that contained 5 mM potassium ferricyande and 5 mM potassium ferrocyanide was used to obtained the voltammograms. The initial potential was 150 mV, the upper potential was 400 mV and the lower potential was –150 mV. The initial scan direction was negative from the initial potential. The scan rate varied form 1000 mV/sec to 25 mV/sec as indicated in Fig. 15.

The 100 μ diameter of the electrodes on the CME9608I ArrayChips is in the size range where the diffusional behavior makes a transition from the semi-infinite linear regime of "macro" electrodes to the radial diffusion regime of "microelectrodes". The series of voltammograms in Figure 15 display the expected transition from the semi-infinite linear transition to radial diffusion as the scan rate is changed. At 1000 mV/sec, the voltammogram has the classic peak shape that is characteristic of semi-infinite linear

diffusion. This peak shaped voltammogram transitions into the polarogram-like shape characteristics of radial diffusion as the scan rate decreases. At 25 mV/sec the voltammogram shape is nearly completely determined by radial diffusion.

The voltammetric response of several different electrodes on an ArrayChip is shown in Figure 16. Addressing different electrodes was accomplished with software that was written in-house to control the ArrayChips during electrochemical experiments. The scan rate was 250 mV/sec and the potential range was –150 mV to 325 mV. The numbers under the voltammograms indicate the coordinates of the electrode used to obtain the voltammogram. This random sampling of electrodes indicates that the electrochemical response of individual electrodes on ArrayChips is consistent and well behaved.

Fig. 17 shows the effect of adding enzyme and substrate to the solution. Initially, the peak current for the electrochemical reduction of ferricyanide (Fe^{3+}) to ferrocyanide (Fe^{2+}) drops immediately and dramatically due to the consumption of ferricyanide by the indoxyl moiety that is produced by enzymatic cleavage of the X-Gal substrate. There is a commensurate increase in the peak current for the electrochemical oxidation of ferrocyanide to ferricyanide. Eventually, all the ferricyanide becomes depleted, as indicated in the final cyclic voltammogram.

There are several ways to monitor the presence of the enzyme tag and to quantitate the electrochemical signal. Peak currents from fast cyclic voltammograms can be monitored and chronocoulometric experiments can be used to re-oxidize the ferricyanide that has been consumed by the substrate. One of the more sensitive methods would be to monitor open circuit potential of the solution and use that as an error signal in a feedback circuit to supply sufficient current to keep the amount of ferricyanide proximate to the electrode constant. This scheme is readily implemented in the hardware and has a very wide dynamic range due to the linearity of the Nernst equation. Alternatively, the open circuit potential could be monitored directly as well as the current and coulombic build up with time.

C) ArrayChip Open Potential Experiments to Monitor β-Galactosidase Activity

Use of individual on ArrayChip electrodes to detect and monitor active b-galactosidase labels using was validated using a homogenous model system. The enzyme reaction scheme used was that outlined in Fig. 14. The electrochemical observable parameter chosen for these initial validation experiments was the open circuit potential at a working electrode. The open circuit potential is a sensitive measure of the ratio of ferrocyanide ion to ferricaynide ion. Oxidation of the indoxyl substrate by ferrocaynide converts the ferrocyanide into ferricyanide and changes this ratio. This process allows the enzymatic reaction to be monitored.

The open circuit potential is related to the ratio of ferro/ferricyanide by the Nernst equation:

$$E = E^{o} - \ln [Fe^{2+}]/[Fe^{3+}]$$

This equation can be transformed to

$$E = E^{o} - \ln [a_{Fe}^{2+}/a_{Fe}^{3+}][\gamma_{Fe}^{2+}/\gamma_{Fe}^{3+}]$$, a = activities; γ = activity coefficients.

Electrochemical systems that obey this equation have a remarkable linear range—often over six orders of magnitude. Because the Nernst equation depends on a ratio, it is reasonably insensitive to the magnitude of the individual concentrations as long as the activity coefficients remain constant. As a result, very small concentrations changes of ferro/ferricyanide can give large open circuit potential shifts. The sensitivity of this assay scales with the concentration of ferro/ferricaynide. Smaller concentrations of the redox couple give a more sensitive assay.

Figure 18 displays the shift in the open circuit potential with time for the homogeneous model assay system. The open circuit cell potential is monitored as the enzyme digests the substrate. A 750 uL aliquot containing 50 mM sodium phosphate buffer (pH7.4), 143 mM ferro/ferricyanide (1:1) and 0.1 units of β-galactosidase enzyme was prepared. The concentration of the enzyme was 50 pM. Fifty uL of a saturated solution of X-Gal substrate (~ 10 mM) was added and the open circuit potential at a single electrode open the ArrayChip was monitored over time. An Ag/AgCl reference electrode was used and both counter and the reference electrode were placed separately into the working solution. The working electrode was on the chip as described previously.

The total shift in the open circuit potential in Fig. 18 is 215 mV. This is a substantial shift in the open circuit potential. The results form his single experiment lead to several other questions. One clearly is what is the limit of detection in the homogenous phase. The second is how does this relate to the limits of detection of enzyme immobilized on the electrode surface.

A more dilute enzyme solution sample was prepared in order to answer some of these equations. A 750 aliquot containing 50 mM sodium phosphate buffer (pH 7.4), 2.9 mM ferro/ferricyanide (1:1) and 0.00356 units of β-galactosidase enzyme was prepared. The concentration of enzyme in this sample was 1.8 pM. Fifty uL of a saturated solution of the X-Gal substrate (~ 10 mM) was added and the open circuit potential at a single electrode on the ArrayChip was monitored over time. An Ag/AgCl reference electrode was used and both counter and the reference electrode were placed separately into the solution. The working electrode was on the chip as described previously.

Figure 19 shows both the shift in the open circuit potential over time for the enzymatic reaction and an intrinsic background shift in the e open circuit potential. The background shift is more than likely due to the oxidation of ferrocyanide by dissolved oxygen. With an enzyme concentration 1.8 pM, the substrate still competes with oxygen to reduce ferrocyanide. The resulting shift of 99 mV is substantially Larger than the background

shift. Removing the dissolved oxygen would produce a more sensitive assay and further lower the limits of detection.

From our present data, the limits of detection for immobilized β-galactosidase (on the chip surface) can be estimated via extrapolation from the homogeneous LODs. The total volume of membrane above a single electrode on an ArrayChip is approximately 8×10^{-12} liter (8 pL). A homogeneous detection limit of 1.8 pM corresponds to approximately 9 enzyme label molecules per electrode site. This is well within the range of sensitivity achieved with sophisticated optical systems.

D) Coulometric Detection of β-Galactosidase with ArrayChip.

The measurement of total charged passed during an enzyme-linked electrochemical experiment is a conventional method for detecting and quantifying enzyme activity. These techniques integrate the current and are usually referred to as coulometric methods. Ultramicro electrodes may have significant advantages for very sensitive coulometric methods because capacitive contributions to the total charge can become negligible.

To demonstrate the detection of β-galactosidase, an ArrayChips was used to detect the enzyme in bulk solution. ArrayChips were immersed phosphate buffer (0.05 M, pH 7.4) containing varying concentrations of β-galactosidase, the substrate X-Gal and ferri/ferrocyanide. An electrode on the ArrayChip was used as the working electrode. The counter and reference electrodes were external to the ArrayChip. A PAR 283 was used to obtain the coulometric data. The applied voltage corresponded to the open circuit potential in the absence of substrate. The results are shown in Figure 20.

As anticipated, the total charged passed passed scales with the concentration of enzyme. The lowest concentration used, 72 pM enzyme, is readily detectable. Quantization of the preliminary coulometric data in Figure 20 is problematic because of the competition of dissolved oxygen and X-Gal in reducing ferricyanide. The effects of dissolved oxygen can be eliminated or compensated for by internal calibration or by displacing oxygen from the system.

E) Immobilization and Detection of β-Galactosidase on the ArrayChip Surface

A checkerboard pattern of biotin was immobilized on the chip surface using the proprietary methods developed at CombiMatrix. Beta-galactosidase that was labeled with a pendant biotin moiety was incubated with streptavidin to form a SA-b-(β-GAL) conjugate. An ArrayChip with immobilized biotin was immersed in a solution of b-galactosidase-streptavidin conjugate for one hour and then washed to remove excess conjugate.

An anti-β-galactosidase was used to ascertain whether β-galactosidase was captured by biotin immobilized within the membrane on the chip surface. The ArrayChip was immersed in a buffer solution containing an anti-β-galactosidase antibody that was

labeled with a Texas Red dye (mouse monoclonal, Sigma Chemical Company). The fluorescence micrograph of the resulting antibody conjugate is shown in Figure 21. This technique clearly enables immobilization of enzyme at selected locations on an ArrayChip.

F) Electrochemical Detection of Immobilized β-Galactosidase on ArrayChips Using Automatic Voltammetric and Amperometric Scanning

CombiMatrix has developed proprietary hardware and software that allows open circuit potentials and current flow to be monitored on ArrayChips. The hardware tools developed internally now allow results from the entire array to be acquired and displayed in minutes. One mode allows the "internal referencing" of the ArrayChip electrodes. This special purpose hardware and software runs on a standard computer platform and enables rapid and flexible electrochemical monitoring. This obviates any third party potentiostats. Electronic circuitry found in many commercial potentiostats is frequently outdated and often of marginal design quality. These shortcomings result in significant limitations when third party potentiostats are used with active semiconductor devices such as ArrayChips.

Additionally, new software has been developed at CombiMatrix that allows the accumulation of all the data (automatically) and display in either digital or pattern diagram (end on display in grey scale). For the digital display, the data may be transferred to Microsoft excel files and the background subtracted before the data is displayed as a 3D plot.

As preliminary validation of the process, these tools were used to detect the presence of β-galactosidase that was immobilized in a checkerboard pattern on an ArrayChip. Unmodified control chips were scanned for potential shifts and currents as controls. No patterns were discerned on the control chips.

Figure 22 displays the output of a potential shift mapping experiment on an ArrayChip with a checkerboard pattern of immobilized β-galactosidase. The software assigns a grey scale value to each electrode that represents the relative potential shift. A grey scale shift to a more white value indicates a shift in the direction expected for enzymatic activity. The observed checkerboard pattern demonstrates enzyme activity on the ArrayChip and corresponds with the locations of immobilized enzyme.

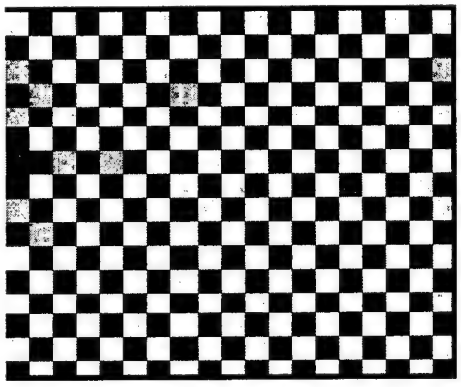

Figure 22

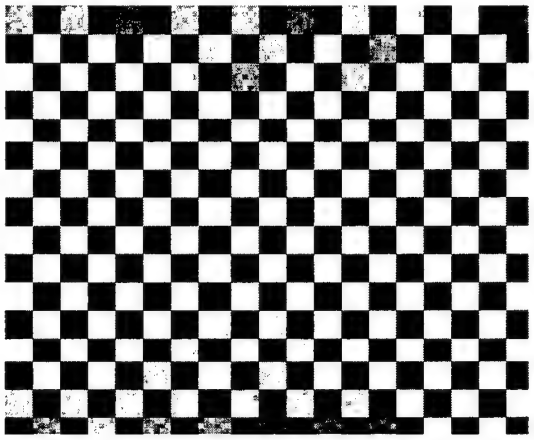

Figure 23

Figure 23 displays the output of an amperometric experiment on an Array Chip with a checkerboard pattern of immobilized β-galactosidase. As in the case of the voltammetric experiment, the software assigns a grey-scale value to each electrode that represents the relative amount of accumulating charge. A grey scale shift to the white indicates a charge accumulation in the direction expected for enzymatic activity. Again, the observed checkerboard pattern demonstrates enzyme activity on the ArrayChip and corresponds with the locations of immobilized enzyme.

Both Figure 22 and Figure 23 were obtained with the same ArrayChip. Both voltammetric and amperometric methods work well for this model system. Experiments are ongoing that will evaluate the relative advantages and disadvantages of the two techniques for detecting enzymatic activity in an assay context.

A better display of the result may be to view a "pattern difference" mapping. This is typically accomplished by looking at a digitally subtracted data (enzyme and substrate vs. no substrate). A section of ArrayChip containing β-galactosidase in a checkerboard data with and with out substrate present is shown in Fig, 24. This data results from a current integration experiment using a unique proprietary internal referencing system.

Figure 25, shows a similar experiment (using the same chip) but based upon the Voltage difference experiment. The results are quite similar, but obviously the internally referenced current experiment shows more promise.

G) Current Chip Development

The CME9608I chip platform can be modified in a facile manner to produce two types of nanode arrays. Both modifications use excimer laser lithography to enable etching of nanometer-scale holes through a dielectric overlaying the electrode pads. The current protocol used by CombiMatrix uses conventional lithography to pattern and etch 100μ vias through this overlaying dielectric layer to expose an electrode (Figure 26). As indicated in Figure 26, a single nanometer-scale via can be etched through to the underlying electrode to produce a nanode. This will produce an array of 1024 individually addressable nanodes. Alternatively, a secondary array of nanometer-scale vias can be etched through to produce an array of nanodes at each CME9608I electrode site (Figure 26). This will produce an array of individually addressable nanode arrays. Other nanode geometries, such as concentric ring nanodes or interdigitated band nanodes, can be contemplated. However, under phase 1 of this contract efforts will focus on arrays of disk nanodes.

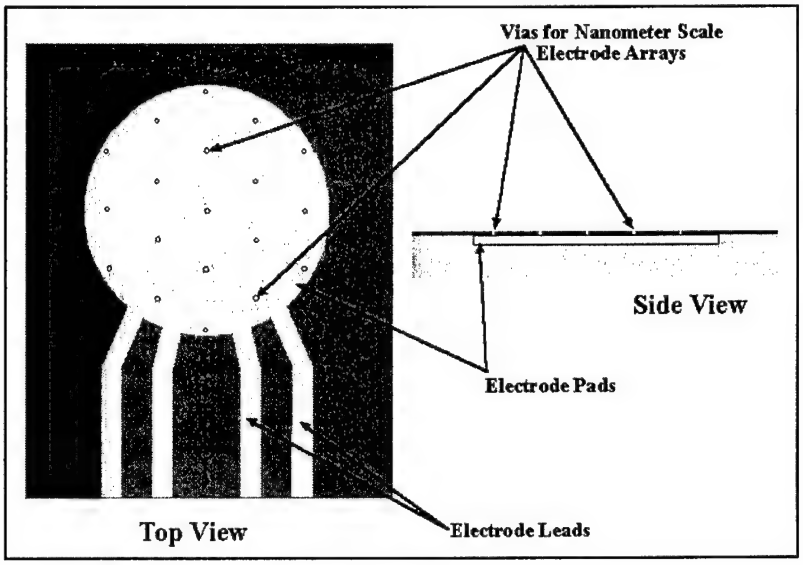

Figure 26

Additional Modes of Detection

In addition to fluorescent and electrochemical detection of immunoassays on the ArrayChip, we began an investigation into the use of colloidal gold conjugates. Colloidal gold can be procured in particle sizes ranging from 5 to 10 nanometers. Proteins can be attached that are useful in immunochemical assays (e.g., streptavidin, antibodies, biotinylated proteins, lectins, and protein A and G). Most of these are commercially available and many of the [proteins can be tagged with fluorophores. Coloidal gold particles can be detected by fluorescence, redox behavior, electrical impedance, optical absorption and optical scattering. Both optical and electrical methods can be used simultaneously.

Some preliminary results using a streptavidin-gold conjugate are shown in Figures 27 and 28. For this experiment, the streptavidin-gold conjugate was captured on a biotin-coated nitrocellulose membrane (0.45 micron pore size; Molecular Devices Corp.). Figure 27 shows the capture of the streptavidin-gold conjugate. Figure 28 shows the control experiment where the SA-Au conjugate had been pre-saturated with biotin before the application to the membrane surface. Thus, the conjugate was unable to bind to the membrane surface.

Additionally we have undertaken several experiments whereby the SA-Au conjugate was captured on the ArrayChip. The results are shown in Fig. 29. The conjugate was detected in a checkerboard pattern under white light. We plan to continue this phase of the investigation during phase II of the project.

Future Studies and Recommendations

One of the first investigations ought to be to broaden our analyte detection base---from lower molecular weight molecules to larger biological moieties. To this end, we need to show that the toxin ricin (Mw 60,000) and the viral particles can also be detected using he ArrayChip.

Another study is the use of antibody—enzyme conjugates, such as Ab-β-galactosidase, can be prepared and used in immunoassays for the detection of analytes of the ArrayChip. Since the β-galactosidase conjugate of antibodies is not commonly available, it needs to be synthesized in several steps. The β-galactosidase can be attached to antibodies using conventional protocols. The heterobifunctional linking agent, succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) will be used. Here, the antibody will be linked through a sulfhydryl moiety and the enzyme through an amino moiety. Immobilized antibody-β-galactosidase conjugate can be monitored via X-Gal substrate/ferricaynide-ferrocyanide coupled redox system.

Another investigation would be to study the use of another enzyme redox system. From the viewpoint of size, the soybean peroxidase may be of interest. The Mw is only 53,000 and it is sold commercially in the activated state (ready for protein conjugation in a single step) at a reasonable price. The only drawback may be to find a mediator that could be used in electrochemical detection process.

The substrate for peroxidase is TMB (3,3',5,5'-tetramethyl benzidine) and hydrogen peroxide peroxide is used (and consumed) as an activator. This redox system is currently under investigation while we wait for the SBIR phase II to begin.

Finally, the ArrayChip needs to be analyte-multiplexed for a host of molecules and biological species.

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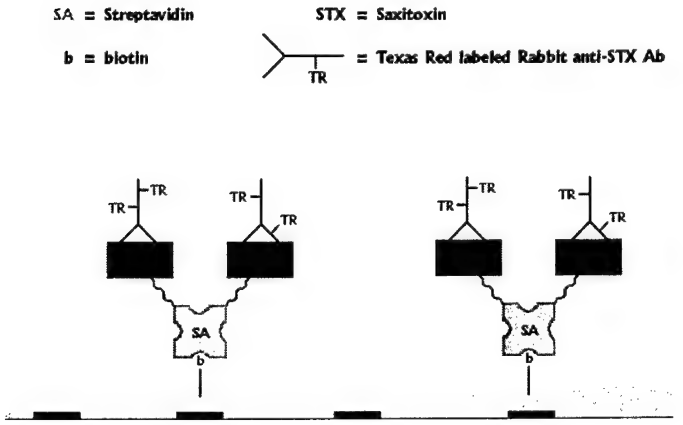

Figure 5. Saxitoxin Immunoassay Scheme

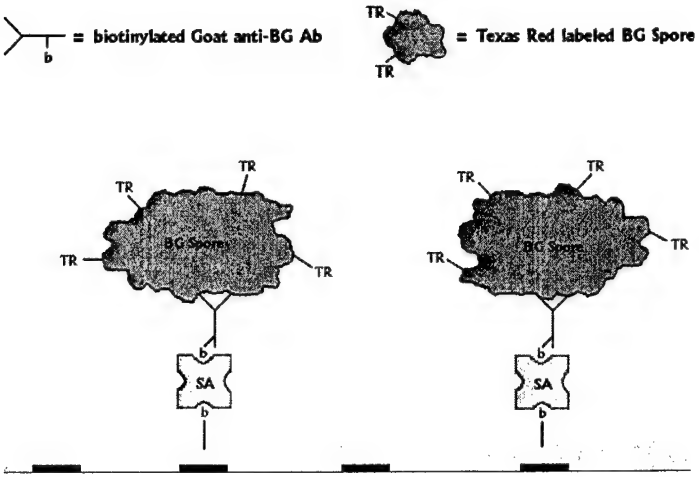

Figure 5. Immunoassay Scheme for TR-BG Spores

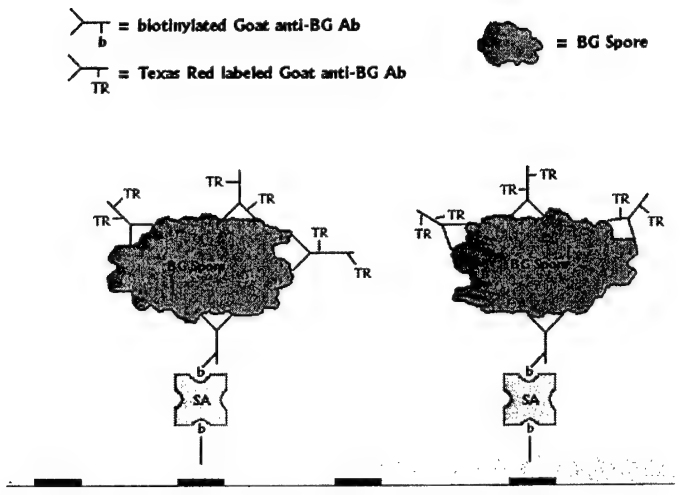

Figure 7. Immunosandwich Assay Scheme for BG Spores

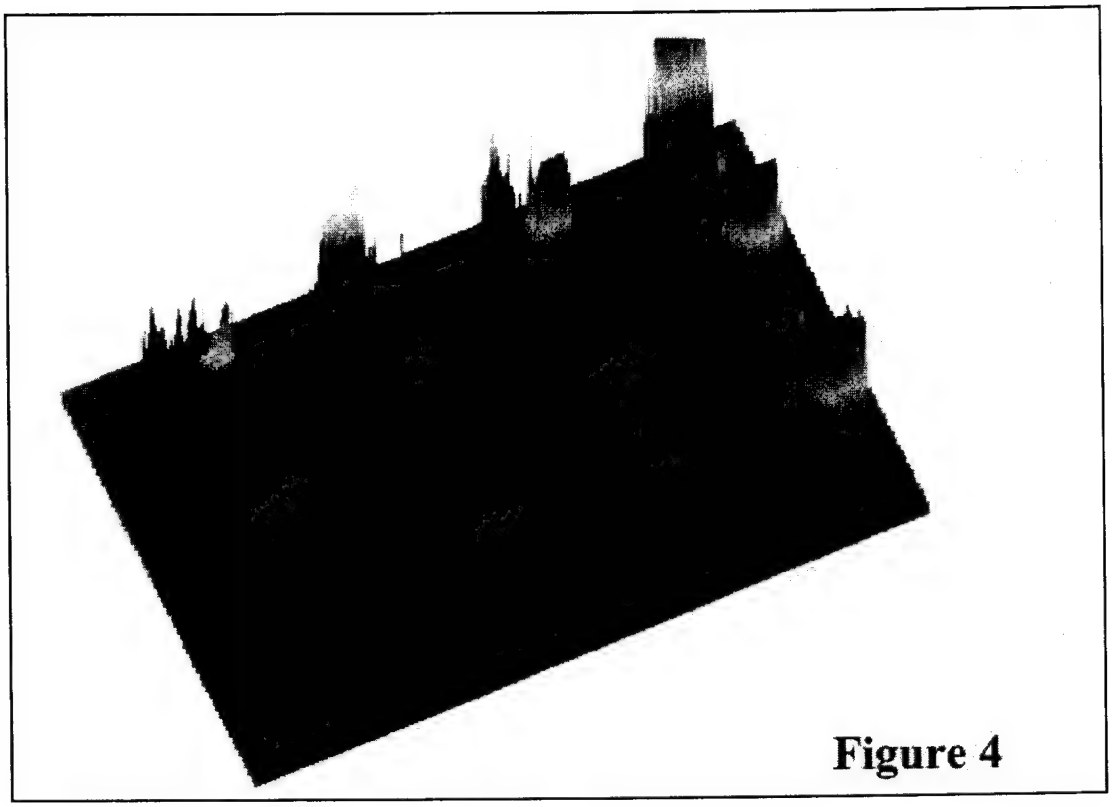

Figure 8. Fluorescence Micorgraph for TR-Labeled α-STX Antibody Bound to the ArrayChip.

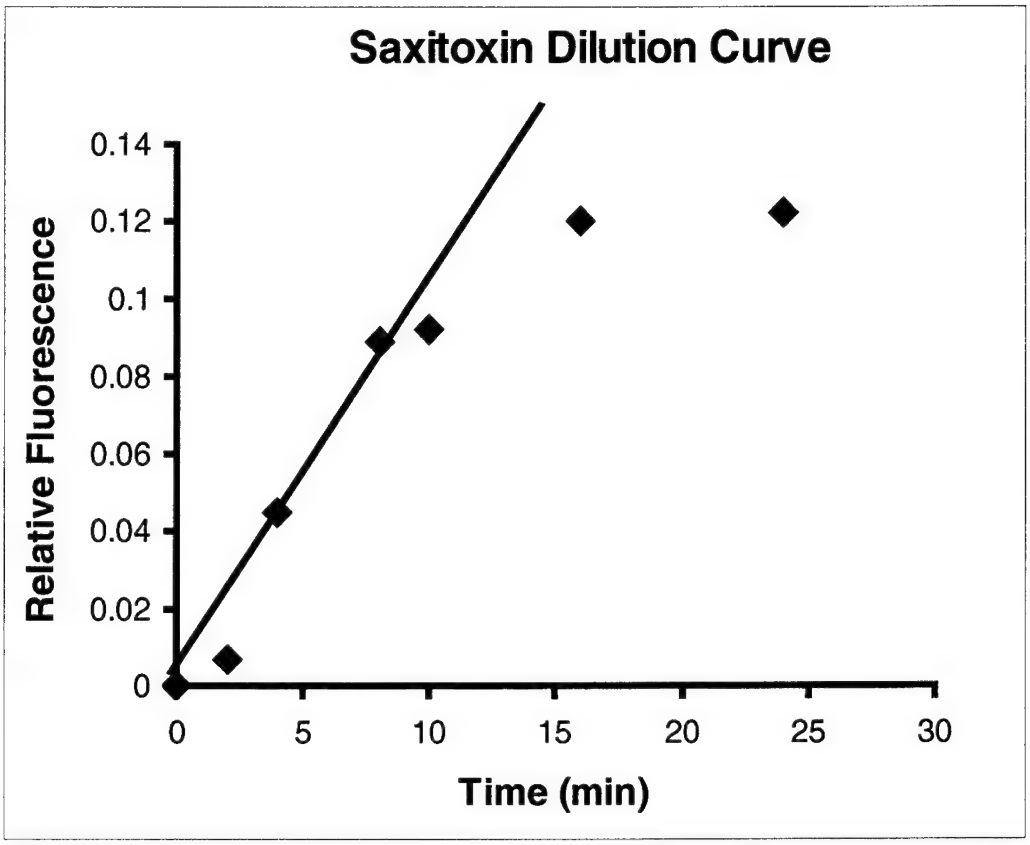

Figure 9. Saxitoxin Dilution Curve

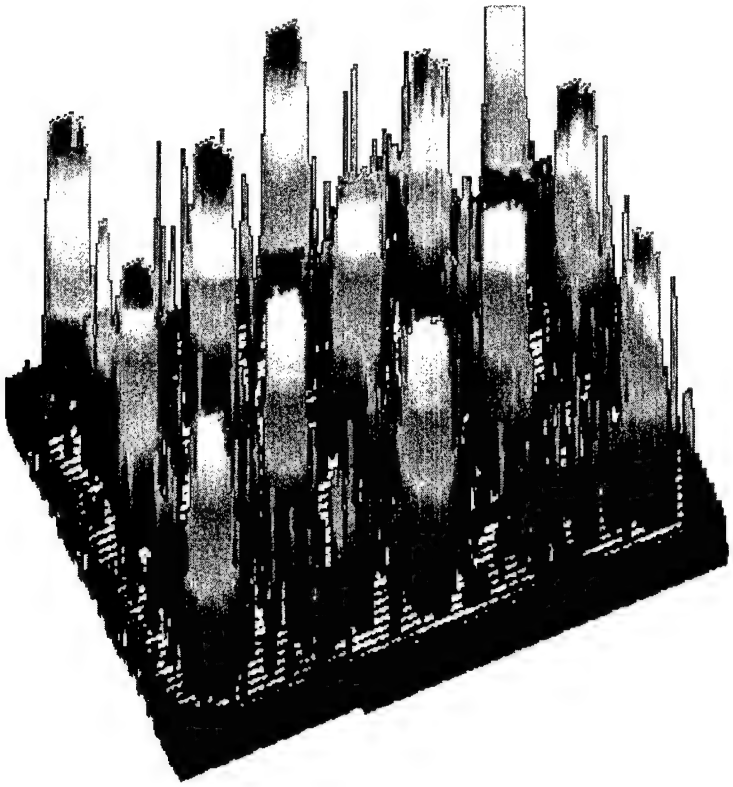

Figure 10. Fluorescence Micrograph for the Capture of TR-BG on the Array Chip.

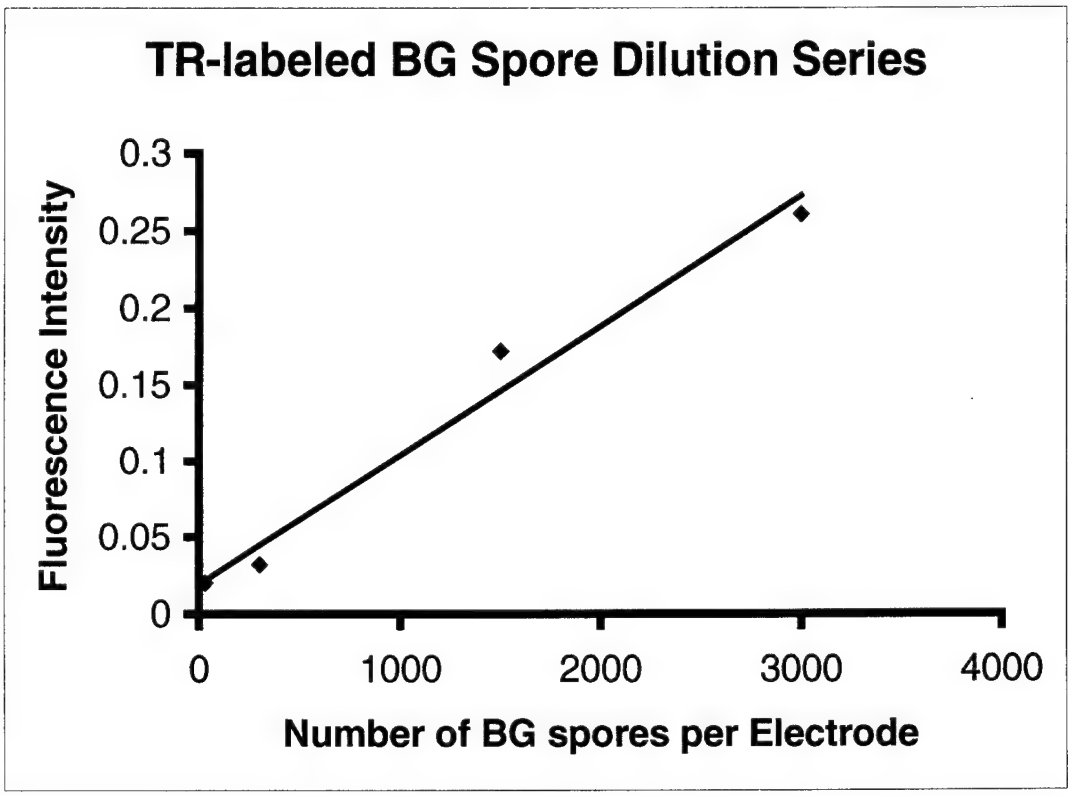

Figure 11. Dilution Series for TR-BG.

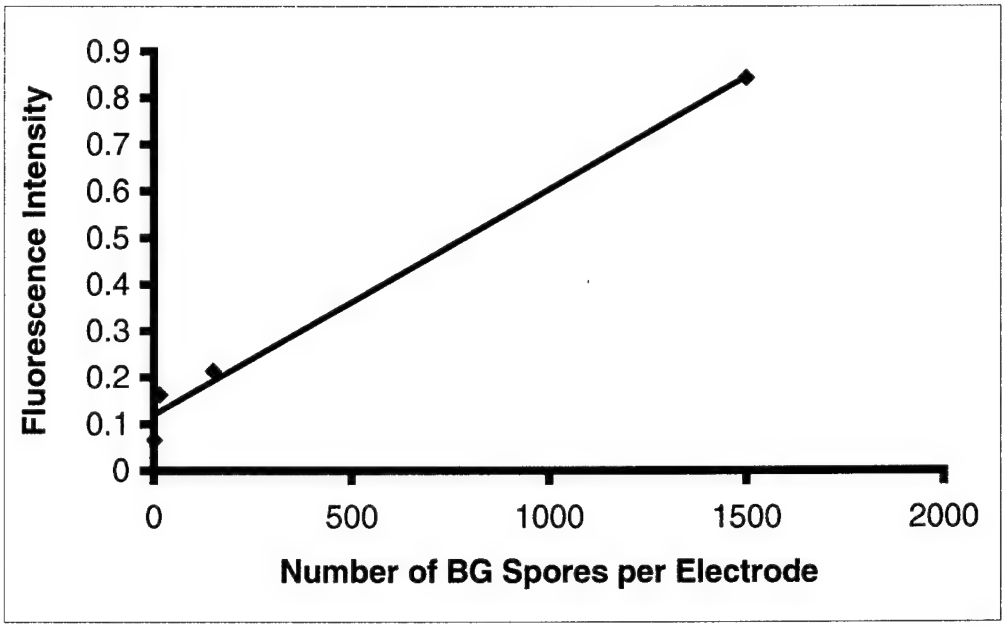

Figure 13. Standard Curve for BG Spores Using an Immunosandwich Assay.

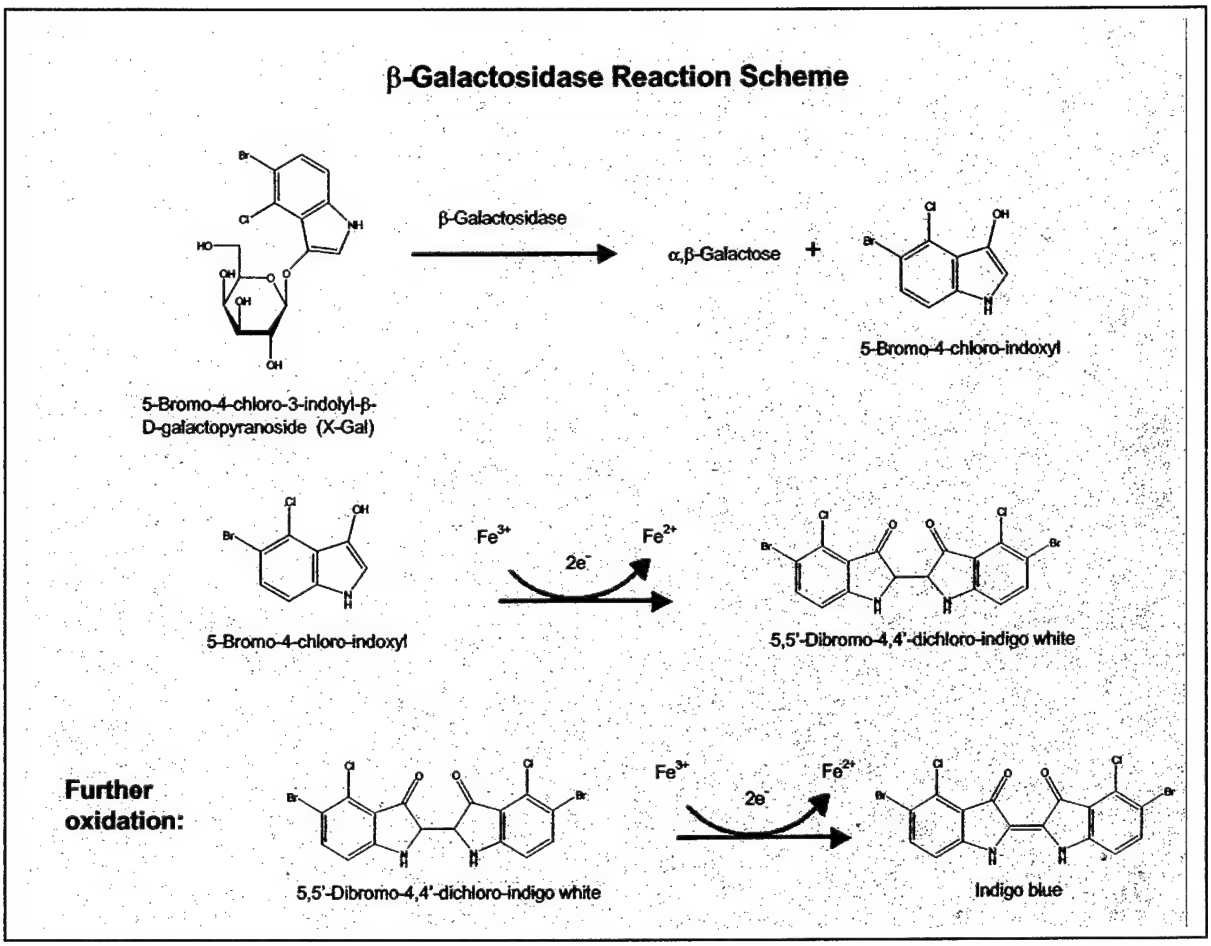

Figure 14. β-Galactosidase Redox Chemistry

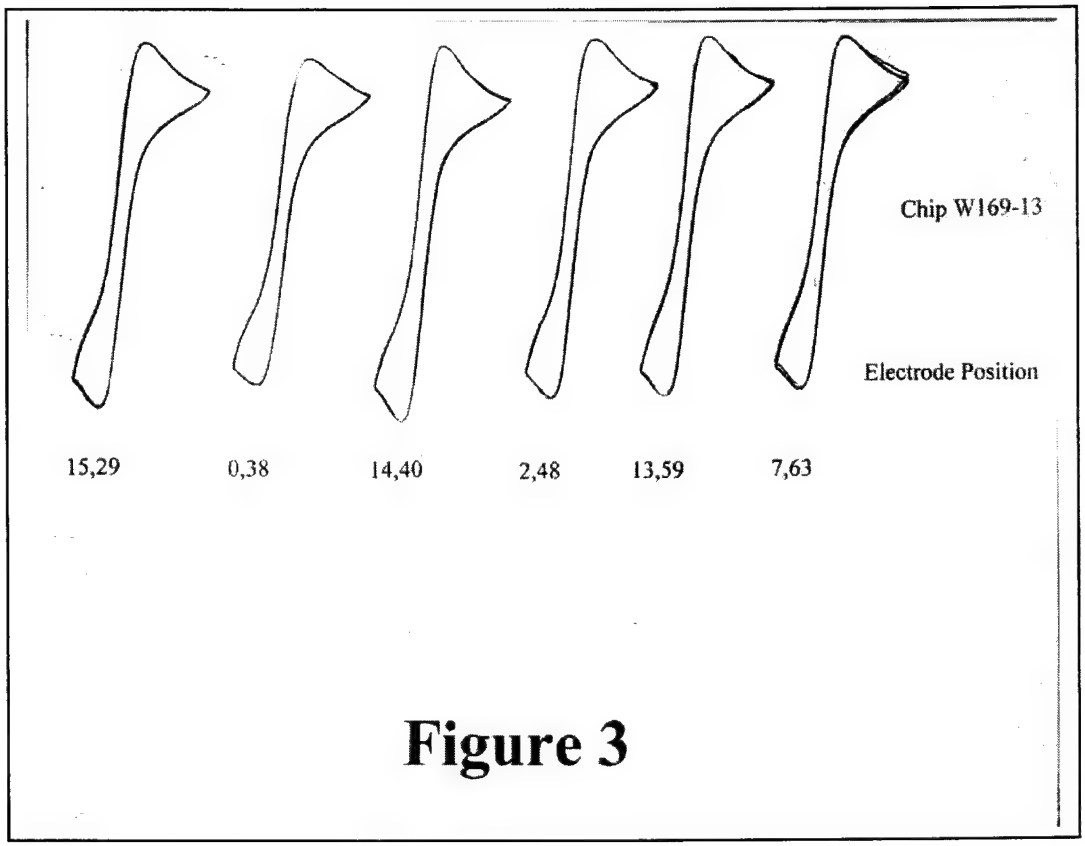

Figure 16. Voltametrix Response of Several Electrodes on the ArayChip.

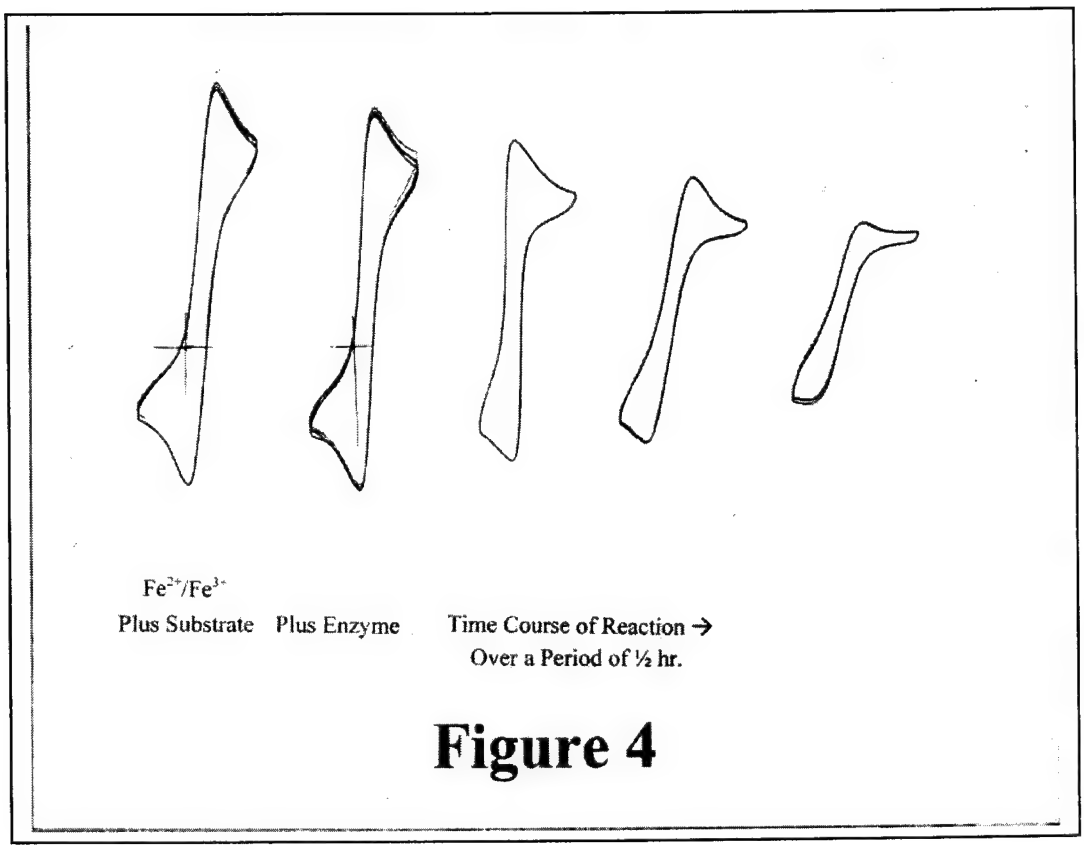

Figure 17. Effects of Enzyme and Substrate on Voltametric Response of an Electrode on the ArrayChip.

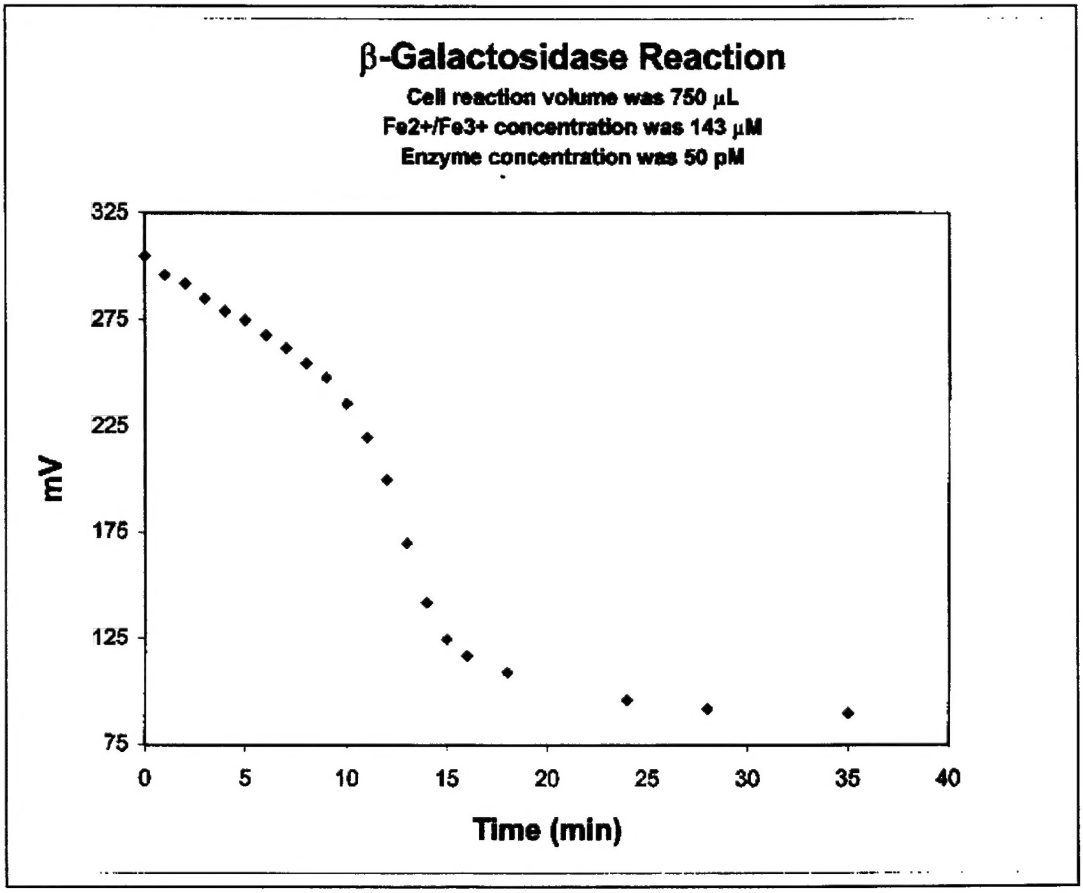

Figure 18. Shift in the Open Circuit Potential on the ArrayChip with Time. Model Enxyme Reaction System.

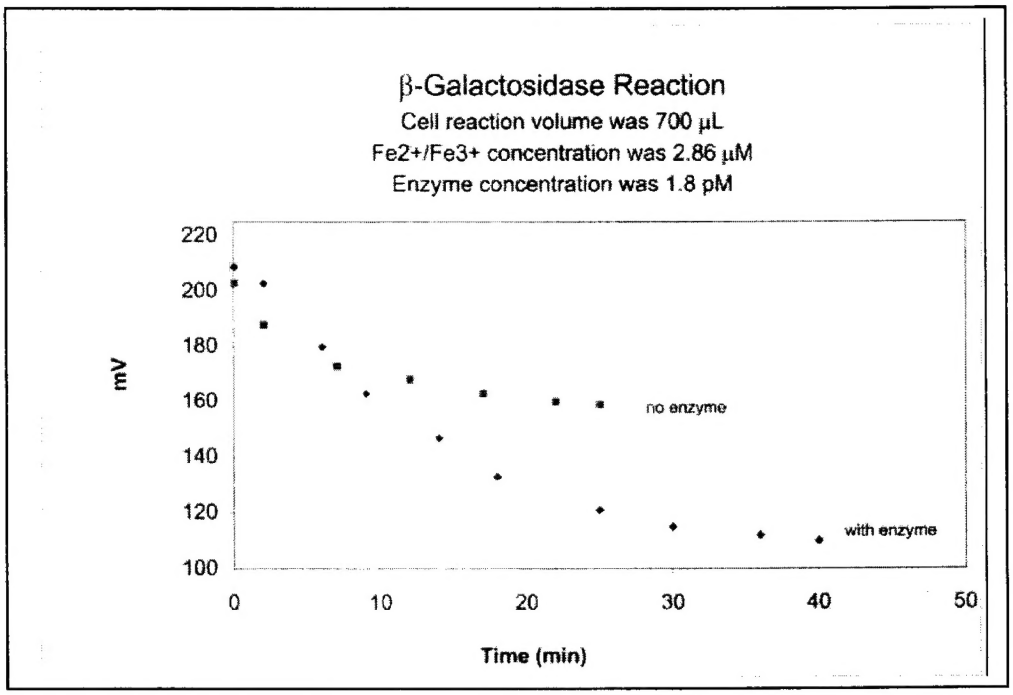

Figure 19. Shift in the Open Circuit Potential on the ArrayChip for a more Dilute Enzyme Model System.

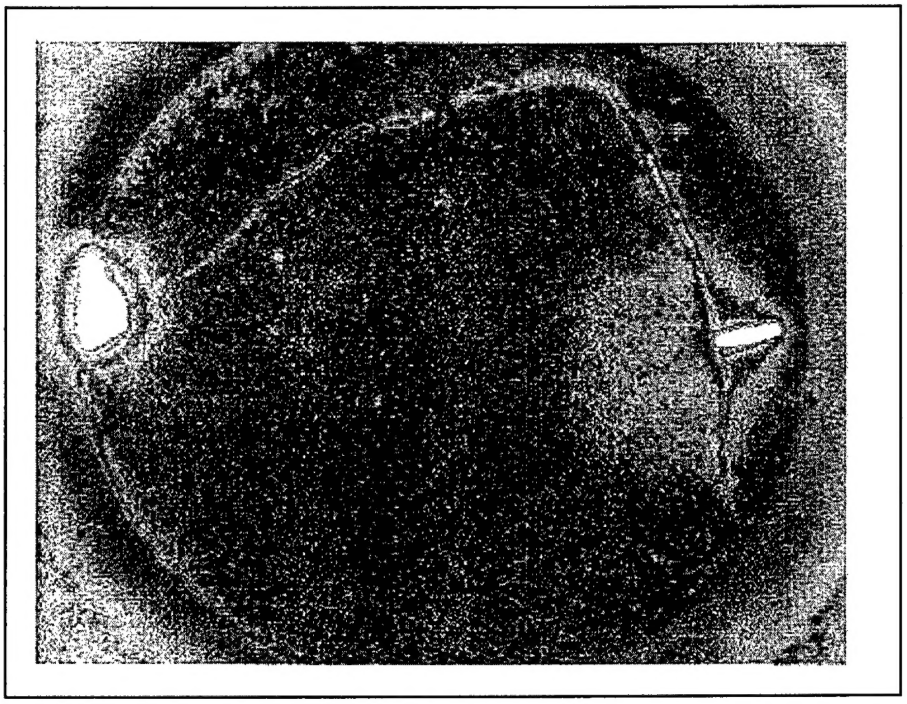

Fig. 27. Capture of Strepavidin-Gold Conjugate on a Biotinylated Nitrocellulose Membrane

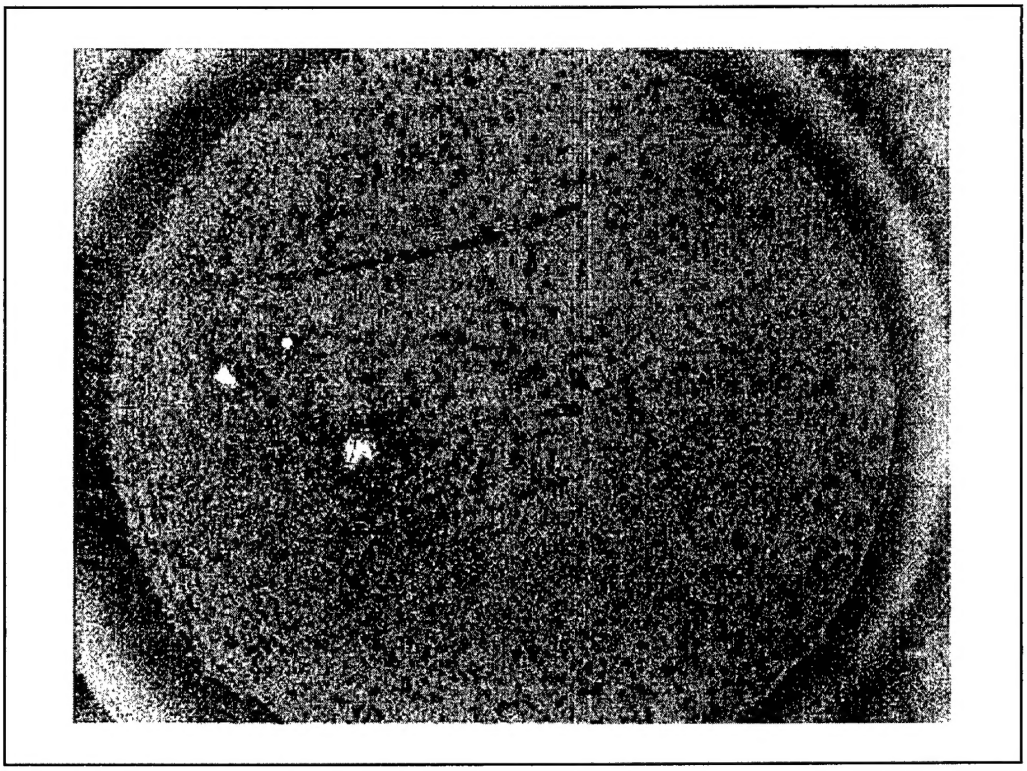

Figure 28. Control Experiment for Fig. 27.